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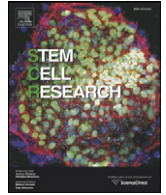
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Lab Resource: Stem Cell Line

Generation of induced pluripotent stem cells (iPSCs) stably expressing CRISPR-based synergistic activation mediator (SAM)

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ABSTRACT

Human fibroblasts were engineered to express the CRISPR-based synergistic activation mediator (SAM) complex: dCas9-VP64 and MS2-P65-HSF1. Two induced pluripotent stem cells (iPSCs) clones expressing SAM were established by transducing these fibroblasts with lentivirus expressing *OCT4*, *SOX2*, *KLF4* and *C-MYC*. We have validated that the reprogramming cassette is silenced in the SAM iPSC clones. Expression of pluripotency genes (*OCT4*, *SOX2*, *LIN28A*, *NANOG*, *GDF3*, *SSEA4*, and *TRA-1-60*), differentiation potential to all three germ layers, and normal karyotypes are validated. These SAM-iPSCs provide a novel, useful tool to investigate genetic regulation of stem cell proliferation and differentiation through CRISPR-mediated activation of endogenous genes.

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Resource table.

Name of stem cell construct	SAM iPSC
Institution	Department of Biomedicine, Aarhus University
Person who created resource	Kai Xiong, Yan Zhou
Contact person and email	Yonglun Luo, alun@biomed.au.dk
Origin	normal human dermal fibroblast
Type of resource	Biological reagent: iPSC stably expressing two CRISPR-based SAM effectors (dCas9-VP64 and MS2-P65-HSF1)
Key transcription factors	<i>OCT4</i> , <i>Sox2</i> , <i>cMyc</i> , <i>Klf4</i>
Link to related literature	NA
Information in public databases	Not available

Resource details

To establish fibroblasts expressing the CRISPR-based synergistic activation mediator (SAM) effectors, normal human dermal fibroblasts (NHDF) were transduced with lentiviral vectors expressing dCas9-VP64 and MS2-P65-HSF1 (Konermann et al., 2014) and selected with blasticidin and hygromycin for 7 days. The survived fibroblasts that expressed SAM were subsequently transduced with a polycistronic lentiviral vector expressing four reprogramming transcription factors (*OCT4*, *KLF4*, *SOX2*, and *C-MYC*) and a red fluorescent marker gene (*dTOMATO*). Four days after transduction, cells were re-seeded to

mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) and cultured in iPSC medium as illustrated in Fig. 1A. ES-like colonies appeared at 12–16 days after transduction (Fig. 1B). Two clones (denoted as SAM iPSC clone 1 and clone 2), with typical ES-like morphology, were manually picked and expanded for further characterizations. First, we validated that both SAM-iPSC lines carry the insertion of SAM expression cassettes in their genome and expressed SAM effectors as shown by PCR-based analysis of genomic DNA and mRNA, respectively (Fig. 1C). Secondly, we investigated whether the reprogramming cassette was silenced in the SAM iPSCs by fluorescent imaging. The polycistronic expression cassette used for reprogramming contains a red fluorescent marker gene (*dTOMATO*), which allows the examination of transgene silencing by fluorescent imaging. Expression of *dTOMATO* was detected at day 14 under reprogramming of SAM fibroblasts into iPSCs. No *dTOMATO* expression was detected in both SAM iPSC lines at passage 7 (Fig. 2), which indicates that the reprogramming cassette has been silenced in the iPSC lines. Thirdly, we characterized the expression of pluripotency genes in the SAM iPSCs by qPCR and immunofluorescence staining. Our qPCR analysis showed that transcription of the endogenous pluripotency genes *OCT4*, *KLF4*, *SOX2*, *LIN28* and *GDF3* in SAM-iPSCs were activated as compared to fibroblasts and at a similar levels as a control iPSC line previously established by us (Kang et al., 2015) (Fig. 3A). Expression of the pluripotency genes *OCT4*, *SSEA4* and *TRA-1-60* were further validated by immunofluorescence staining (Fig. 3B). Fourthly, we characterized the differentiation potential of these two SAM iPSC clones. Embryoid bodies (EB) were generated through suspension culture in ultra-low attachment plate followed by spontaneous differentiation for 21 days. Our results showed that both SAM iPSC lines can give rise to the three germ layers by staining with marker antibodies

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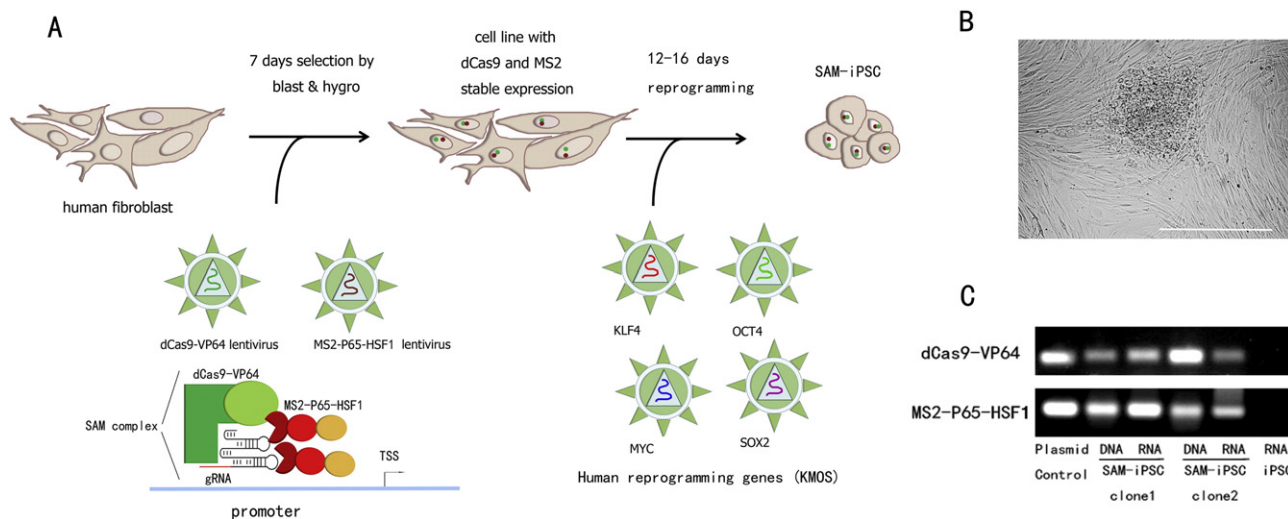


Fig. 1. Generation of SAM iPSCs. A. Illustration of SAM complex and flow-chart of SAM-iPSC generation. B. Typical ES-like colonies of SAM-iPSCs emerged 14 days after transduction. Scale bars: 400 μ m. C. PCR detection of integration and expression of SAM activator components in two SAM-iPSC clones.

against beta-III tubulin (TUJ1) for ectoderm, smooth muscle actin (SMA) for mesoderm, and alpha-fetoprotein (AFP) for endoderm (Fig. 4A). Finally, karyotyping analysis confirmed that each SAM iPSC clone maintained the normal euploid karyotype after reprogramming (46, XX) (Fig. 4B). Our results collectively confirmed that the two novel iPSC clones express the CRISPR synergistic activation mediators, are positive for the expression of the investigated pluripotency genes, capable of giving rise to all three germ layers, and karyotypically normal. These two SAM-iPSC clones provide a novel, useful tool to investigate genetic regulation of stem cell proliferation and differentiation in the future.

Methods and materials

Lentivirus production

HEK293T cells (Life Technologies) were cultured in DMEM medium (LONZA) supplemented with 10% FBS (Sigma), 1% Glutamax (Life technologies) and 1% penicillin/streptomycin (Life Technologies). 1×10^7 cells were seeded on 15 cm plates in 20 ml medium 1 day before transfection. Cells were transfected on the next day when they had reached 80–90% confluency. 31.5 μ g of lentiviral plasmid containing the gene of interest, 31.5 μ g of pMDGP-Lg/pRRE, 9.07 μ g of pMD2.G and 7.26 μ g of pRSV-REV were diluted in 1089 μ l ddH₂O and mixed with 121 μ l of 2.5 M calcium chloride solution and 1210 μ l of $2 \times$ HBS solution. After incubation at room temperature for 20 min, the solution mixture was added drop-wise directly to cells. Medium was renewed 24 h after transfection. Virus supernatant was harvested twice at 48 h and 72 h post transfection, and then filtered with a 0.45-mm PVDF filter (Millipore). Concentrated lentiviral supernatants were generated when needed by ultracentrifugation for 2 h at 25,000 rpm, 4 $^{\circ}$ C, and stored at -80° C.

Lentiviral transduction and SAM-iPSC generation

Normal human dermal fibroblasts (anonymous donor, generously provided by Prof. Thomas G. Jensen from the Department of Biomedicine, Aarhus University) were cultured in DMEM (LONZA) supplemented with 10% FBS (Sigma, 1% Glutamax (Life Technologies) and 1% penicillin/streptomycin (Life Technologies) and passaged at a 1:3 ratio when cells reached 70–80% confluency. Cells were transduced with crude lentivirus containing dCas9-VP64 (a gift from Feng Zhang's lab, Addgene #61425) and MS2-P65-HSF1 (a gift from Feng Zhang's lab, Addgene #61426). 7.5×10^5 cells were plated in a T75 flask one day before virus transduction. 15 ml of crude virus with 8 μ g/ml polybrene (Sigma) were added to the cells. 48 h after transduction, lentiviral supernatant were replaced by fresh medium. Selection reagents were added immediately (blasticidin and hygromycin, Life Technologies). The working concentration of drug selection was optimized by kill curve assays giving the following optimized concentration: 2 μ g/ml blasticidin and 200 μ g/ml hygromycin. Medium was replaced every other day for 7 days. Surviving cells were passaged and cultured in medium with 1 μ g/ml blasticidin and 100 μ g/ml hygromycin. One day before lentiviral reprogramming, selected cells were detached with 0.05% Trypsin-EDTA (Life Technologies) and replated in 6-well plates

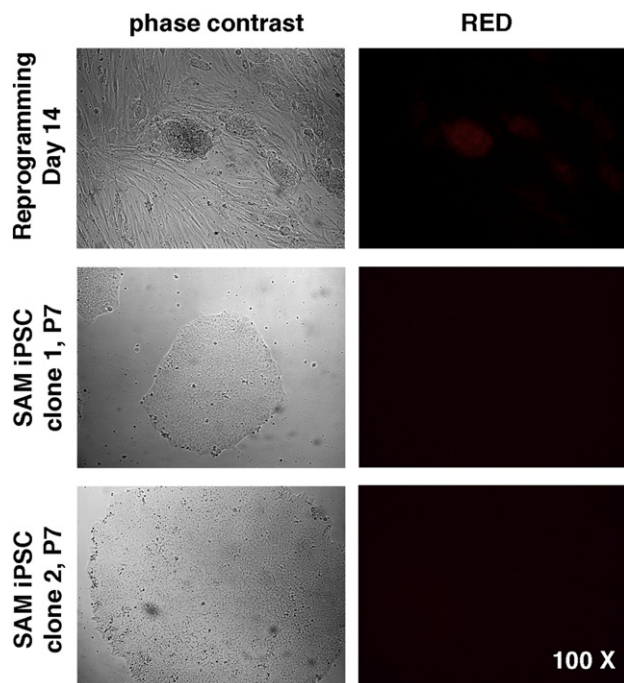


Fig. 2. Evaluation of reprogramming cassette expression and silencing based on fluorescent imaging. Representative phase-contrast and red fluorescent images taken from iPSCs clones during reprogramming (at day 14 after transduction), and the two SAM iPSC clones at passage 7. Magnification: 100 \times .

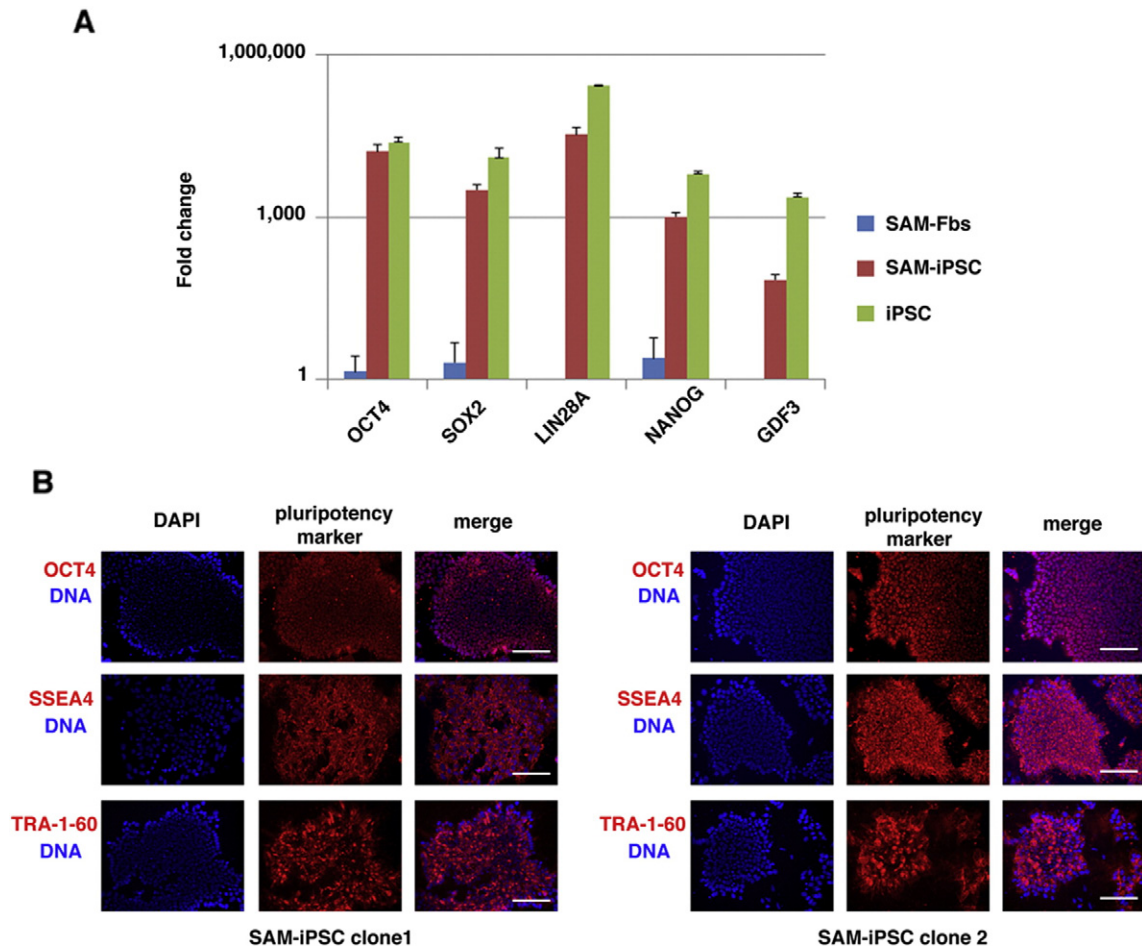


Fig. 3. Gene expression analysis of pluripotency genes in SAM iPSCs. **A.** Quantitative reverse-transcriptase PCR (qRT-PCR) expression analyses of cDNA from SAM-iPSCs. Standard iPSCs made from normal fibroblasts without integration of SAM components were used as a positive control in studies of the endogenous pluripotency genes *OCT4*, *SOX2*, *LIN28A*, *NANOG* and *GDF3*. Relative expression, normalized with GAPDH, was shown as fold change ($2^{-\Delta\Delta C_t}$) compared with SAM-fibroblasts. **B.** Immunofluorescence staining of pluripotency markers *OCT4*, *SSEA4* and *TRA-1-60* in two SAM-iPSC clones. Scale bars: 200 μ m.

at a density of 9×10^4 cells per well. The cells were then transduced by 2 ml crude lentivirus expressing *OCT4*, *SOX2*, *KLF-4* and *MYC*. Medium was changed every other day. Four days after transduction, transduced cells were harvested and re-seeded in 6-well plates coated with mitomycin C inactivated MEFs at 1:3 splitting ratio. 24 h later, culture medium was changed to iPSC medium, consisting of Knockout DMEM, 20% knockout serum replacement (Life Technologies), 2 mM Glutamax (Gibco), 1% non-essential amino acids (NEAA, Life Technologies), 1% penicillin/streptomycin (P/S, Sigma), and 10 ng/ml of bFGF (Life technologies). The iPSC medium was changed every day. Two ES-like clones were isolated and cultured in E8 medium in a feeder-free culturing system with 0.5 μ g/ml blasticidin and 50 μ g/ml hygromycin. The SAM-iPSCs were passaged manually or by means of 0.5 mM EDTA.

Quantitative PCR analysis

Total RNA was isolated from SAM-iPSCs using RNeasy Mini Kits (Qiagen) following the manufacturer's instructions. Relative RNA expression levels were quantified by reverse transcription using iScript cDNA Synthesis Kit (Bio-Rad) (Promega, San Luis Obispo, US) and quantitative PCR (qPCR) using SYBR Green I Master Kit (Roche). Primers are listed in Table 1. Data were analyzed by the $\Delta\Delta C_t$ method: Target Ct values were normalized to GAPDH Ct values, and fold change in target gene expression was determined by comparison to fibroblasts. Quantitative PCR analysis was performed in triplicate for each sample.

Immunofluorescence staining

Cells were washed once with PBS and fixed for 20 min with 400 μ l of 4% paraformaldehyde. After fixation, cells were washed with PBS containing 2% FBS, and then permeabilized for 30 min with 0.5% Triton-X100. Subsequently, cells were blocked for 30 min with 5% donkey serum, and incubated with primary antibodies diluted in PBS containing 0.1% Triton-X100 and 1% donkey serum. Primary antibodies were the following: *OCT4* (Abcam, ab27985, goat polyclonal, 1:300 dilution), *SSEA4* (Abcam, ab16287, mouse monoclonal, 1:300 dilution) and *TRA-1-60* (Abcam, ab16288, mouse monoclonal, 1:300 dilution) at 4 °C overnight. The following day, cells were washed 3 times with PBS containing 2% FBS and afterwards incubated with secondary antibodies for *OCT4* (Alexa-594 donkey anti goat, 1:500), *SSEA4* (Alexa-594 goat anti mouse, 1:500), *TRA-1-60* (Alexa-594 goat anti mouse, 1:500) respectively at room temperature for 2 h. Cells processed identically at all steps but without adding primary antibodies were used as control.

Embryoid body (EB) formation and differentiation

Embryoid body-mediated spontaneous differentiation was performed as previously described (Rasmussen et al., 2014) with small modifications. Briefly, iPSCs were dissociated with PBS-EDTA (0.5 mM) for 5 min at 37 °C, and 100,000 cells were re-plated per well onto 24-well ultra-low attachment plates (Corning), and cultured in E8 medium for one week with medium change daily. EBs with round

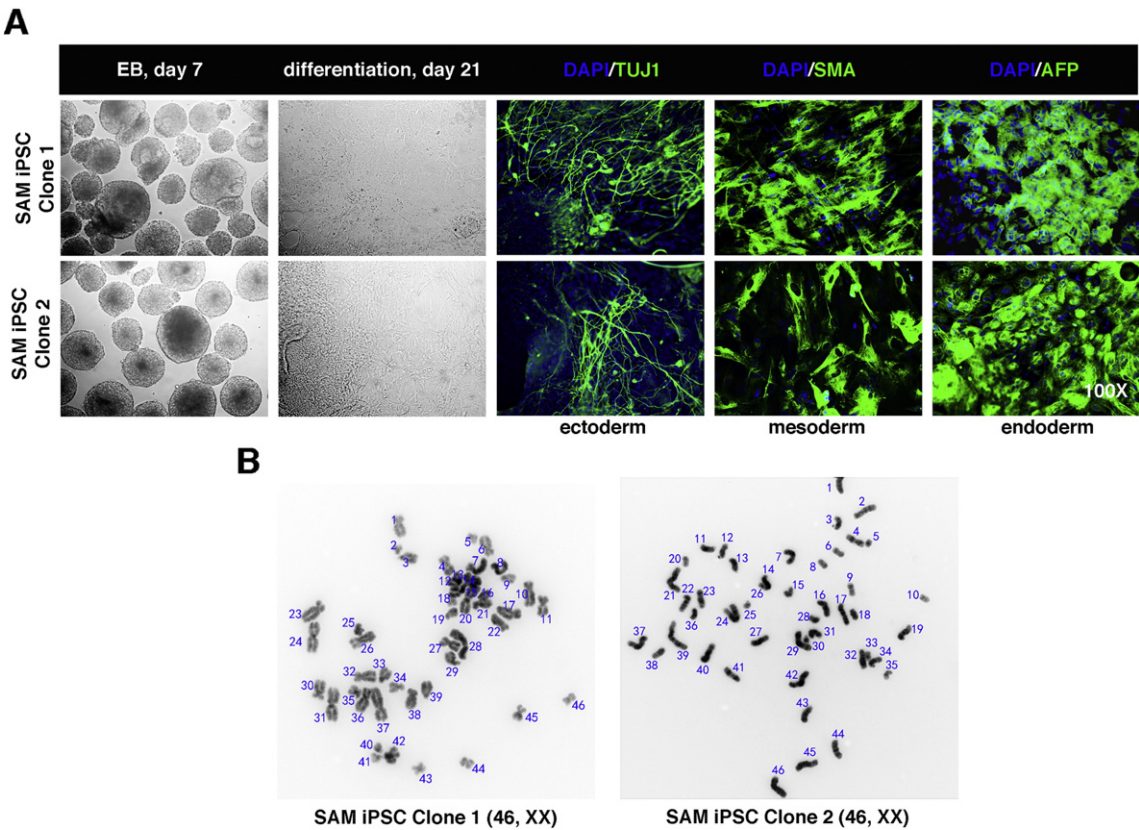


Fig. 4. EB-mediated three germ layer differentiation and karyotyping. A. Embryoid bodies were generated from both SAM iPSC clones (day 7 Ebs after culture in E8 medium and ultra-low attachment plate) and subjected to spontaneous differentiation into all three germ layers for 21 days (phase contrast image of the differentiated cells). Three germ layers are detected by antibodies against beta-III tubulin (TUJ1, green) for ectoderm, smooth muscle actin (SMA, green) for mesoderm, and alpha-fetoprotein (AFP, green) for endoderm. DAPI nuclear DNA counterstaining (blue) was also performed. B. Representative normal euploid karyotype of the two SAM iPSC clones.

and aggregated morphology were transferred to 0.1% gelatin coated 4-well chamber slides and cultured with differentiation medium (high glucose DMEM medium supplemented with 10% FBS, 2 mM L-glutamine and 1% pen/strep) for three weeks. The medium was changed every 2–3 days. The differentiated cells were fixed with 4% PFA for immunostaining analysis against TUJ1 for ectoderm (A25532, life technologies, 1:500 dilution), AFP for endoderm (A008, DAKO, 1:500 dilution) and SMA for mesoderm (M0851, DAKO, 1:500 dilution). Nuclei were visualized by DAPI staining for 10 min.

Karyotyping analysis

Karyotyping analysis was conducted using DAPI-based banding as described previously (Zou et al., 2013). Briefly, iPSCs were cultured until they reach 70% confluent in a 35 mm dish. KaryoMAX® Colcemid™ Solution (Gibco, 15212) were added to the culture medium with a final concentration of 0.1 µg/ml and incubated for 2.5 h at 37 °C. The iPSCs were harvested by 0.5 mM EDTA-PBS, followed by swollen with

pre-warm 0.56% KCL for 15 min and fixed with freshly-made and cold fixative solution (methanol: acetic acid, 3:1) for 30 min on ice. Small droplets of fixed iPSCs were spread onto glass slides and allowed to dry without disturbing. The slides were mounted DAPI antifade solution and analyzed by DAPI banding. Metaphases were examined and analyzed by Quips CGH software.

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Table 1
Primer used in this study.

Gene	Forward	Reverse
hOCT4	CCTCACTTCACTGCACTGTA	CAGGTTTTCITTCCTAGCT
hSOX2	CCCAGCAGACTTCACATGT	CCTCCCATTTCCCTCGTTTT
hLIN28	AGTAAGCTGCACATGGAAG	ATTGTGGCTCAATTCTGTGC
hNANOG	TCCAACATCCTGAACCTCAG	ACCATTGCTATTCTTCGGCC
hGDF3	TCTCCCAGACTTATGCTACG	AGTAGAGGAGCTTCTGCAGG
hGAPDH	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC
dCas9-VP64	GAAGAGAAATGCTGGCCTCTG	CTCGATGATCTCGTCCAGGT
MS2-P65-HSF	CGAGGGGACTCTGAGTGAAG	AGCATTTGGTTCGGCTGTACT

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